Estradiol mediates the long-lasting lung inflammation induced by intestinal ischemia and reperfusion

Short Title: Intestinal IR alters lung homeostasis

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Key words: intestinal ischemia and reperfusion, 17β-estradiol, lung, inflammation, cytokines, uric acid.

Abstract

Background: Lung inflammation is one of the main consequences of intestinal ischemia reperfusion (intestinal IR) and, in severe cases, can lead to acute respiratory distress syndrome (ARDS) and death. We have previously demonstrated that estradiol exerts a protective effect on lung edema and cytokine release caused by intestinal IR in male rats.

Materials and Methods: We investigated the role of estradiol on the generation of IL-1β, IL-10, VEGF, and CINC-1 in a female rat model of intestinal IR. Blood and bone marrow leukocytes were also quantified. Seven-days-ovariectomized (OVx) rats were subjected to intestinal IR by occlusion of the superior mesenteric artery for 45 min. After re-perfusion of the tissue for 2 hours, the rats were sacrificed. Lung tissue was collected, cultured for 24 hours and assayed.

Results: We observed a significant increase in serum levels of IL-10, CINC-1, uric acid and circulating, but not bone marrow, leukocyte numbers. In addition, intestinal IR induced a significant increase in the *ex-vivo* lung levels of IL-1β, IL-10 and VEGF. Treatment with 17β-estradiol before the induction of intestinal IR prevented the systemic release of IL-10, CINC-1 and uric acid, but it did not affect the leukocytosis. In addition, 17β-estradiol significantly prevented the *ex-vivo* release of IL-1β and VEGF from lung tissue.

Conclusions: We demonstrated that intestinal IR interferes with lung homeostasis, priming the tissue to generate pro-inflammatory mediators for at least 24 hours post-ischemia. Furthermore, our data confirm that the inflammatory responses caused by intestinal IR are estradiol mediated.

Introduction

Intestinal ischemia and reperfusion (IR) is a life-threatening medical disorder characterized by the systemic release of a wide spectrum of inflammatory mediators and oxygen radicals, thus activating the innate immune response and resulting in local and remote organ dysfunction [1, 2, 3]. During ischemia, low tissue oxygenation leads to an energy imbalance whereby the Adenosine Tri-Phosphate/Adenosine Monophosphate (ATP/AMP) ratio is shifted to increased levels of AMP that, in turn, are catabolized to hypoxanthine and uric acid [4]. The release of uric acid is elevated after an ischemic event and hence, is considered an indicator of low oxygenation of the gut tissue [5]. Uric acid is also a well-recognized stimulus of the innate and adaptive immune response [6, 7] .

Acute lung inflammation is a major consequence of intestinal IR and is characterized by leukocyte migration, increased microvascular permeability and release of pro-inflammatory mediators. In severe cases, this inflammation can lead to acute respiratory distress syndrome (ARDS), multiple organ failure and death. Despite advances in pharmacological therapies, the mortality rate of ARDS is still high (around 40%), indicating a need for a better understanding of its underlying mechanisms [8, 9]. We have previously demonstrated that ovariectomized rats develop a significantly higher lung microvascular permeability and neutrophil influx after intestinal IR in comparison to female rats with intact ovaries. Interestingly, the replenishment of these ovariectomized rats with 17β-estradiol prevented the increase of lung microvascular permeability by a nitric oxide-mediated mechanism, but it did not affect neutrophil migration into the lung [10]. These results suggest that endogenous estrogens protect female rats against some of the inflammatory repercussions of intestinal IR. Moreover, we have also shown that treatment with 17β-estradiol protects male rats against lung injury induced by intestinal IR, confirming the important role of female sex hormones in this process [11]. Estradiol has also been shown to be protective in experimental models of ischemia-reperfusion in other organs such as heart [12, 13], brain [14, 15], kidney [16], and liver [17, 18] .

In this study, we sought to investigate the role of estradiol on the systemic release of inflammatory mediators after two hours of intestinal reperfusion in female rats, and on the *ex-vivo* release of mediators by lung tissue after 24 hours of culture. To this purpose, we measured estradiol modulation of the release of serum and tissue cytokines, the number of leukocytes in the bone marrow and blood compartment, and the generation of uric acid in an ovariectomized rat model.

Materials and Methods

Animals and study groups

Seven days after ovariectomy, female Wistar rats (180–220 g) from our Department´s animal facilities were assigned to three groups: (1) Sham IR: rats surgically manipulated but not submitted to intestinal IR; (2) IR: rats submitted to intestinal IR and (3) IR+E: rats treated with estradiol and 24 hours later submitted to intestinal IR. These studies were approved by the Animal Care Committee of the Institute of Biomedical Sciences, University of Sao Paulo, following the guidelines of the National Council of Animal Experimentation that regulates animal research according to Brazilian Federal Law (Report no. 111/10/03 - 2013).

Ovariectomy (OVx)

Rats were anesthetized with isoflurane (2%) and an incision was made on the lower part of the abdomen; the ovaries were identified and removed free from the adherent tissue. After OVx, the animals received a single dose of Pentantibiotic® (570 mg/kg, i.m.), Tramadol® (5 mg/kg, i.p.) and Paracetamol® (530 μg/ml, in the drinking water *ad libitum* for three days). The effectiveness of the ovariectomy was verified 7 days later by cell patterns in vaginal smears and only rats with smears compatible with diestrus phase (elevated number of leukocytes) were used in these experiments. In addition, at the end of each experiment the weight of the uterus was measured and compared to uteri from rats with intact ovaries. Animals with significant loss in weight of their uterus in comparison to controls were considered successfully ovariectomized**.**

Intestinal IR model

After 7 days of OVx, the rats were anesthetized with ketamine-xylazine (100 and 20 mg/kg, respectively, intraperitonially) and the superior mesentery artery was occluded for 45 min., after which the clip was removed and sutures performed to close the abdomen. After two hours of reperfusion all animals were euthanized by an over dose of ketamine-xylazine.

Ex vivo culture of lung tissue

Lungs were perfused through the pulmonary artery with 20 mL of phosphate buffer salt solution (PBS, 30ml/min). Samples of lung parenchyma were cut into small pieces and incubated in 24-well plastic microplates (four pieces per well) containing Dulbecco's Modified Eagle Medium (DMEM) at 37° C in a humidified atmosphere with 5% CO_2. Aliquots of the supernatants were collected after 24 hours of incubation for cytokine quantification.

Quantification of IL-1β, IL-10, VEGF and CINC-1

The levels of interleukin (IL) 1β, 10, vascular endothelial growth factor (VEGF) and of cytokine-induced neutrophil chemoattractant 1 (CINC-1) were determined in serum by Milliplex® commercial ELISA kit (Merck Millipore, EUA). The concentration of IL-1β, IL-10 and VEGF were quantified using Duo Set® commercial ELISA kit (R&D System, USA) in aliquots of DMEM collected from lung tissue cultures. All assays were conducted following the specifications of the manufacturer.

Circulating blood leukocytes and bone marrow cell numbers

Peripheral blood leukocytes were collected from the tail vein before and after induction of intestinal IR and mixed in Turk's solution (1:20). Total cells from bone marrow were obtained by flushing the femur with a syringe containing 10 mL of PBS. The suspension was then placed in a solution of violet crystal 0.2% (1:10). Cells were quantified using optical light microscopy with a Neubauer chamber.

Quantification of serum levels of uric acid

Uric acid concentrations in serum were determined colorimetrically, using a commercial kit according to the specification of the manufacturer (Bioclin/Quibasa, Minas Gerais, Brazil).

Pharmacological treatment

Ovariectomized rats (OVx) were treated with a single subcutaneous injection of 17β-estradiol (280 µg/kg – Sigma-Aldrich, Darmstadt, Germany) diluted in vehicle (Sunflower Oil, Liza, Brazil) 24 hours prior to the induction of intestinal IR.

Statistical analysis

Data are expressed as mean ± SEM. Comparisons between groups were made by one way, ANOVA followed by Tukey post-test using the GraphPad Prism (version 6.0). Values of $P < 0.05$ were considered significant.

Results

Cytokines levels in serum

To analyze the systemic effects of intestinal IR we determined the levels of cytokines in serum from blood samples obtained from ovariectomized rats following 2 hours of intestinal reperfusion. As shown in figure 1, there was a significant increase in IL-10 and CINC-1 levels for IR group of rats in comparison to Sham IR rats (Figure 1, panels B and D). Pre-treatment with 17β-estradiol significantly prevented the increase of these mediators in the serum. Systemic release of IL-1β and VEGF was not significantly altered by intestinal IR or 17β-estradiol (Figure 1, panels A and C).

Cytokines levels in ex-vivo culture of lung tissue

Following 2 hours of intestinal reperfusion, lung samples were collected from the euthanized, ovariectomized rats. Figure 2 shows the levels of cytokines released by lung tissue after 24 hours of culture in DMEM. Panels A, B and C demonstrate that lung collected from IR rats produced significantly higher levels of IL-1β, IL-10 and VGEF in comparison to samples from Sham IR animals. *In vivo* treatment of ovariectomized rats with 17β-estradiol prior to induction of intestinal IR significantly prevented the release of IL1-β and VGEF but did not affect the release of IL-10 by lung tissue 24 hours later.

Effect of 17β-estradiol treatment on leukocytes count following intestinal IR

As shown in Figure 3 (panel A), intestinal IR induced a significant increase in circulating leukocytes in rats that was not affected by pre-treatment with 17β-estradiol. When compared to Sham IR rats, the number of leukocytes in the bone marrow was not significantly affected by intestinal IR or pre-treatment with 17β-estradiol (Figure 3, panel B).

Effect of 17β-estradiol treatment on uric acid levels following intestinal IR

Figure 4 shows that intestinal IR significantly increased the levels of uric acid in the IR group compared to Sham IR rats. Pre-treatment with 17β-estradiol significantly prevented the increase of

systemic uric acid after intestinal IR, with uric acid levels for IR+E rats similar to values found in Sham IR animals.

Discussion

In previous studies, we have shown that the lungs of male rats generate inflammatory mediators in response to intestinal IR and that this response can be modulated by treatment with 17β-estradiol after to the induction of ischemia [11]. In this study, we have investigated the effects exerted by estradiol on the lung and systemic inflammation caused by intestinal IR in female ovariectomized rats. We show that intestinal IR induced a significant increase in both the levels of circulating IL-10 and CINC-1 as well as lung tissue levels of IL-1β, IL-10 and VEGF, measured after 24 hours of culture.

Experimental and clinical evidence indicates that IL-1β is an important mediator in several models of systemic inflammation [19], sepsis [20, 21], trauma hemorrhagic shock [22], and intestinal IR [23]. In fact, IL-1β induces mobilization of leukocytes between compartments and is a marker of inflammasome activation [24, 25]. In our OVx rat model, intestinal IR resulted in a significant increase in IL-1β levels in cultured lung tissue, but not in the systemic circulation. Furthermore, this increase was inhibited by pre-treatment with 17β-estradiol, indicating that this response is modified by the female sex hormone.

Similar to IL-1β, when compared to sham IR animals, intestinal IR caused an increase in VEGF levels measured in cultured lung tissue, but not in the circulation. Again, this effect is modified by 17β-estradiol. Mura et al. (2006) [26], using an intestinal IR model in mice found high levels of VEGF in the lung of both sham and intestinal IR groups after 4 hours of intestinal reperfusion. VEGF has been shown to be important in leukocyte recruitment into the lung during inflammation [27, 28, 29] but its role in microvascular permeability is also well recognized. Interestingly, VEGF increases vascular permeability by a nitric oxide mediated mechanism [30]. We have previously reported that

estradiol downregulates lung vascular permeability induced by intestinal IR in OVx rats by a mechanism dependent on nitric oxide activity [10].

Taken together, these data demonstrate that intestinal IR causes changes in lung tissue homeostasis that persists for at least 24 hours after intestinal reperfusion. More importantly, our *ex-vivo* lung cultures strongly suggest that the ability of the lung tissue to continuously produce pro-inflammatory mediators is not dependent on further stimulation from the system or the gut. Lung tissue culture is a well-established method for measuring *ex-vivo* ongoing release of mediators from the tissue. However, there are limitations with this method which should be taken into consideration. Among these are the use of a single time-point and the exclusion of other endogenous factors that could influence the response *in vivo*.

Moreover, this study suggests that estradiol differentially regulates the systemic and lung release of cytokines induced by intestinal IR, confirming previous observations of the protective role of estrogen in lung injury in male rats. Thus, we hypothesized that the low levels of estradiol induced by the removal of the ovaries increases the generation/release of inflammatory mediators such as IL-1β and VEGF, worsening the lung injury induced by intestinal IR. Supporting our hypothesis, estradiol did not affect the release of the anti-inflammatory cytokine IL-10 by the lung tissue, possibly released as an attempt to control the inflammatory response induced in OVx rats [31].

In this study, we have also found that at in reduced levels of estrogen, intestinal IR caused a robust circulating leukocytosis that was not accompanied by significant changes in bone marrow cell numbers. We have also found an increased level of CINC-1 in serum, a potent neutrophil chemotactic agent [32] that was significantly inhibited by pre-treatment with estradiol. We have previously reported that estradiol fails to down regulate the migration of neutrophils into the lung of female rats. However, this study suggests that an effect of estradiol on circulating chemotactic factors may occur [11]. It is important to highlight that even though estrogen does not regulate leukocyte migration into the lung, CINC 1 in the circulation may be having its effects elsewhere[33].

In our model, we found a significant increase in the levels of uric acid in the circulation after 2 hours of reperfusion, indicating a decrease in tissue oxygenation [5]. Furthermore, we found that estradiol had an inhibitory effect on the intestinal IR-induced increase in uric acid, suggesting an interaction between this hormone and uric acid generation. These data allow us to speculate that downregulation of the expression of danger signals may be one of the mechanisms by which estrogen protects females against lung damage induced by intestinal IR. Supporting our findings, Mumford et al. (2013) [34] demonstrated that in pre-menopausal women, estradiol reduces uric acid levels, which is associated with the protective effects of estradiol on cardiovascular diseases in this cohort. Noteworthy, Gasse et al. (2009) [35] also established a correlation among increased levels of uric acid and activation of inflammasome NLRP3 in a model of acute lung injury in mice.

In conclusion, our study indicates that intestinal IR induces an inflammatory status in the lung that remains for a long period after the mesenteric reperfusion has been re-established. This status is characterized by the release of pro-inflammatory mediators and danger signals. Our study also presents strong evidence that this persistent inflammatory status in the lung, observed after intestinal IR, is downregulated by estrogen in female OVx rats.

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Legends of the figures

Figure 1. Cytokine quantification in serum collected 2h after intestinal IR: IL-1β (A), IL-10 (B), VEGF (C) and CINC-1 (D). Sham IR were rats manipulated but not subjected to intestinal IR (open bar), IR were subjected to intestinal IR (grey bar) and IR+E were subjected to intestinal IR and treated with estradiol (280 µg/kg, s.c) 24 hours before ischemia induction (black bar). Data was analyzed by one-way ANOVA and expressed as mean ± SEM. In panel B: *p<0.05 *vs* Sham IR; ^{o}p<0.05 *vs* IR. In panel D: *p<0.05 *vs* Sham IR; ^{o}p<0.05 *vs* IR.

Figure 2. Cytokine quantification in supernatants of lung tissue collected at 24h of incubation in DMEM. IL-1β (A), IL-10 (B) and VEGF (C) Lung samples were collected 2h after intestinal IR. Sham IR were rats manipulated but not subjected to intestinal IR (open bar), IR were subjected to intestinal IR (grey bar) and IR+E were subjected to intestinal IR and treated with estradiol (280 µg/kg, s.c.) 24 hours before ischemia induction (black bar). Data was analyzed by one-way ANOVA and expressed as mean ± SEM. In panel A: *p<0.05 *vs* Sham IR; ^{o}p<0.05 vsIR. In panel B: *p<0.05 *vs* Sham IR. In panel C: * p<0.0001 *vs* Sham IR; ^{o}p<0.05 *vs* IR.

Figure 3. Cell count determined in peripheral blood (A) and bone marrow (B) collected 2h after intestinal IR. Sham IR were rats manipulated but not subjected to intestinal IR (open bar), IR were subjected to intestinal IR (grey bar) and IR+E were subjected to intestinal IR and treated with estradiol (280 µg/kg, s.c) 24 hours before ischemia induction (black bar). Data was analyzed by one-way ANOVA and expressed Data expressed as mean ± SEM from 6 to 8 animals. In A: *p<0.05 *vs* Sham IR.

Figure 4. Quantification of Uric Acid in serum collected 2h after intestinal IR. Sham IR were rats manipulated but not subjected to intestinal IR (open bar), IR were subjected to intestinal IR (grey bar) and IR+E were subjected to intestinal IR and treated with estradiol (280 µg/kg, s.c.) 24 hours before ischemia induction (black bar). Data was analyzed by one-way ANOVA and expressed as mean ± SEM from 6 to 8 animals. *p<0.05 *vs* Sham IR; ^{o}p<0.05 *vs* IR.

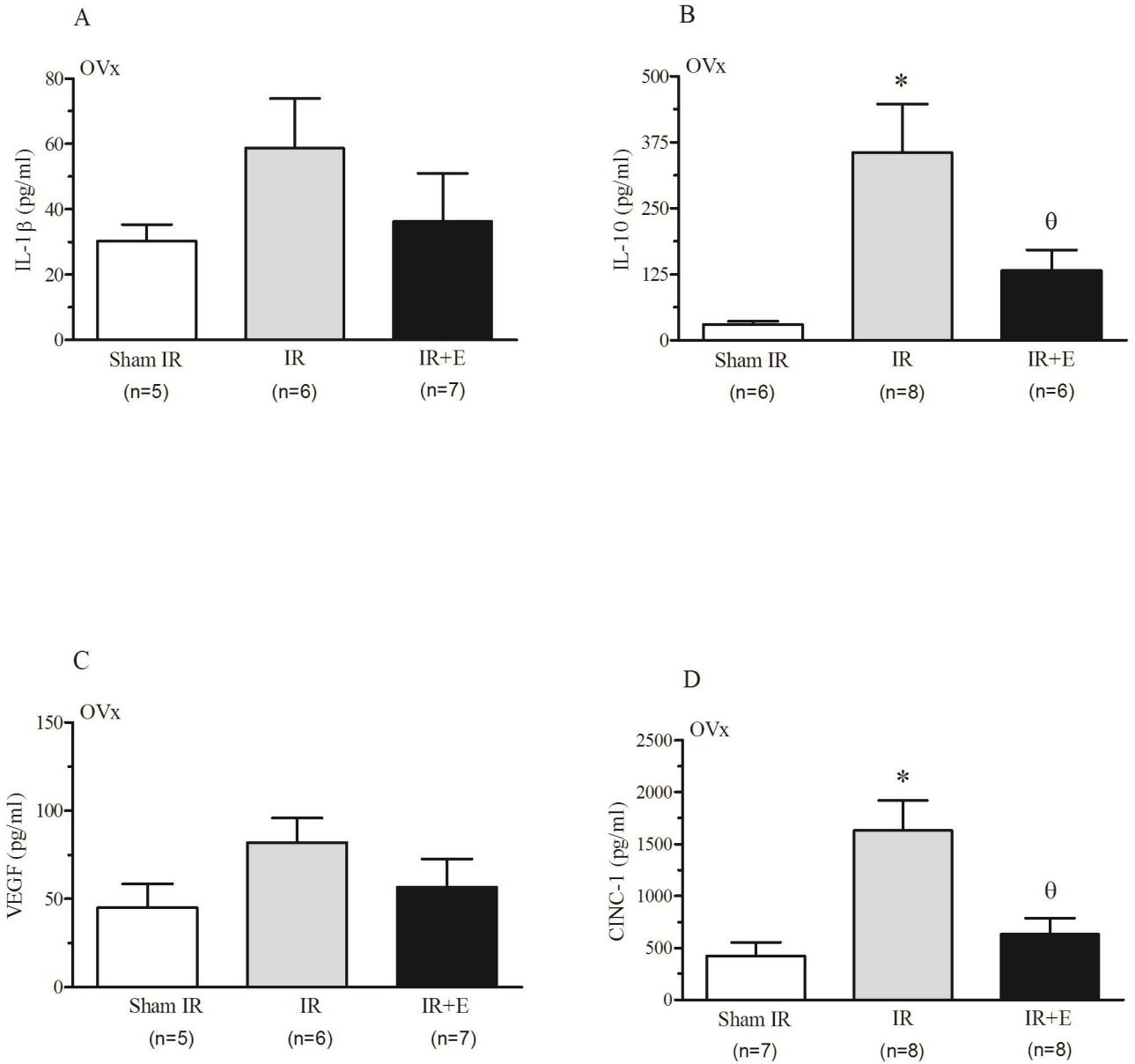

Figure 1. Fantozzi et al. 2017

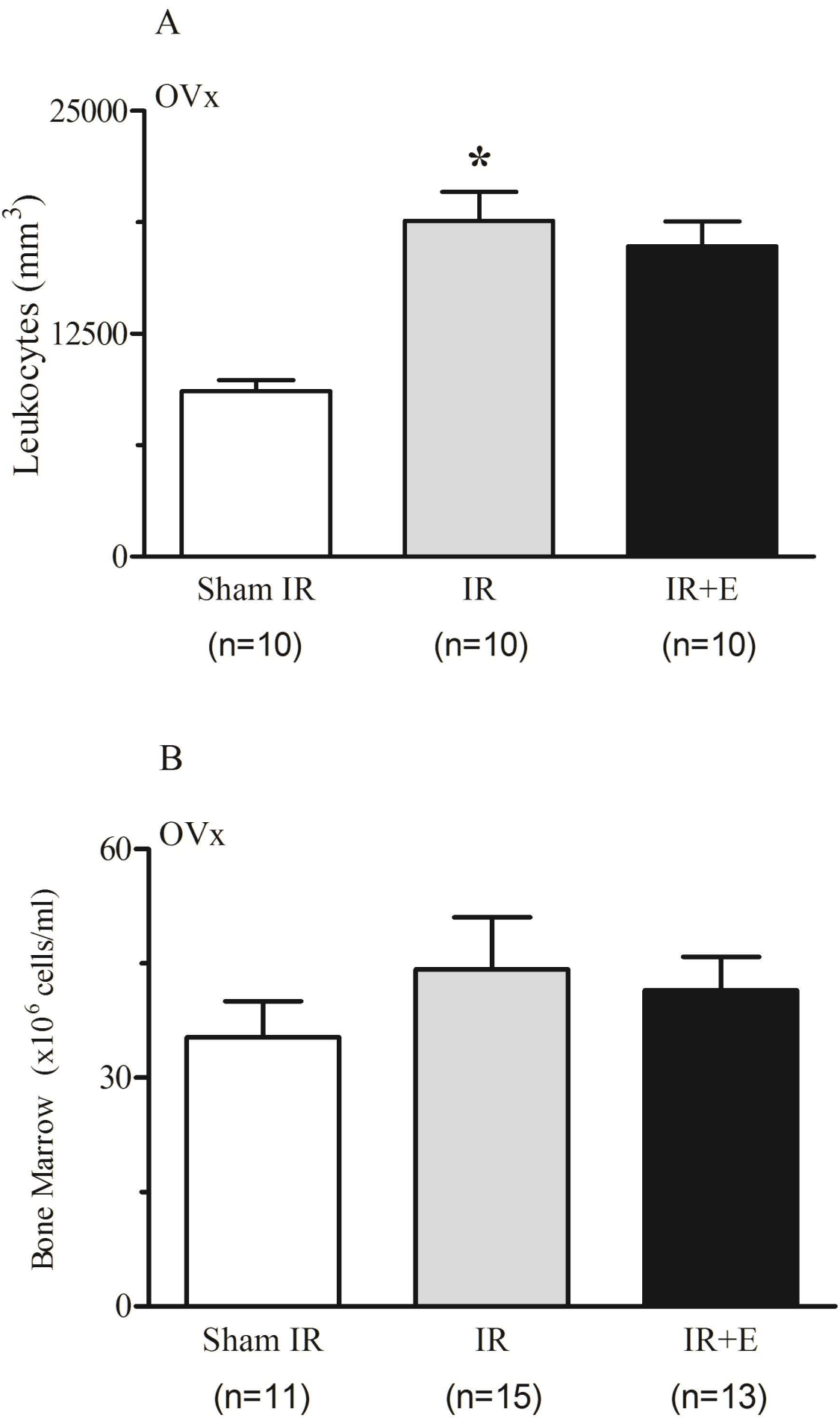

Figure 3 Fantozzi et al., 2017

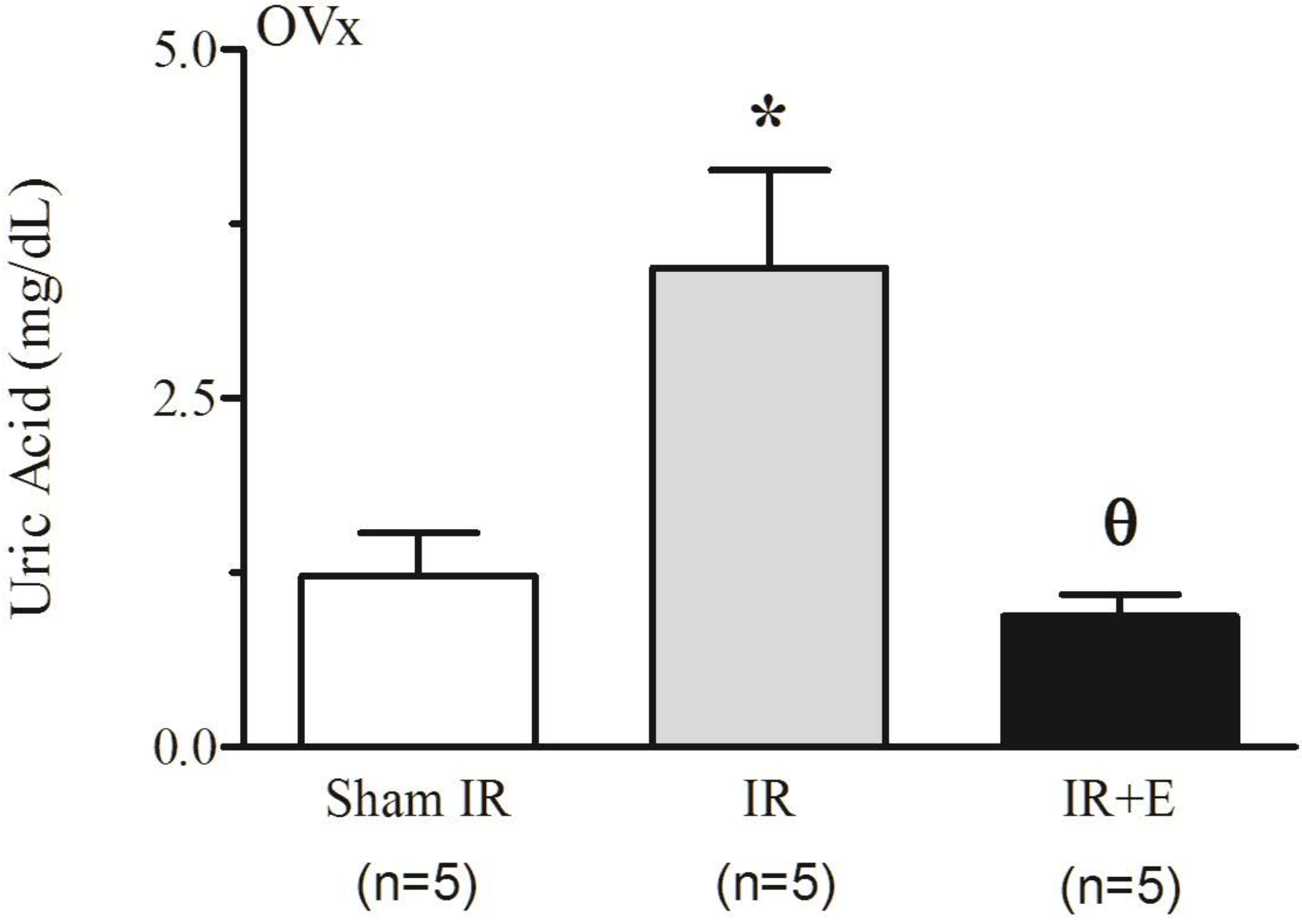

Figure 4. Fantozzi et al. 2017

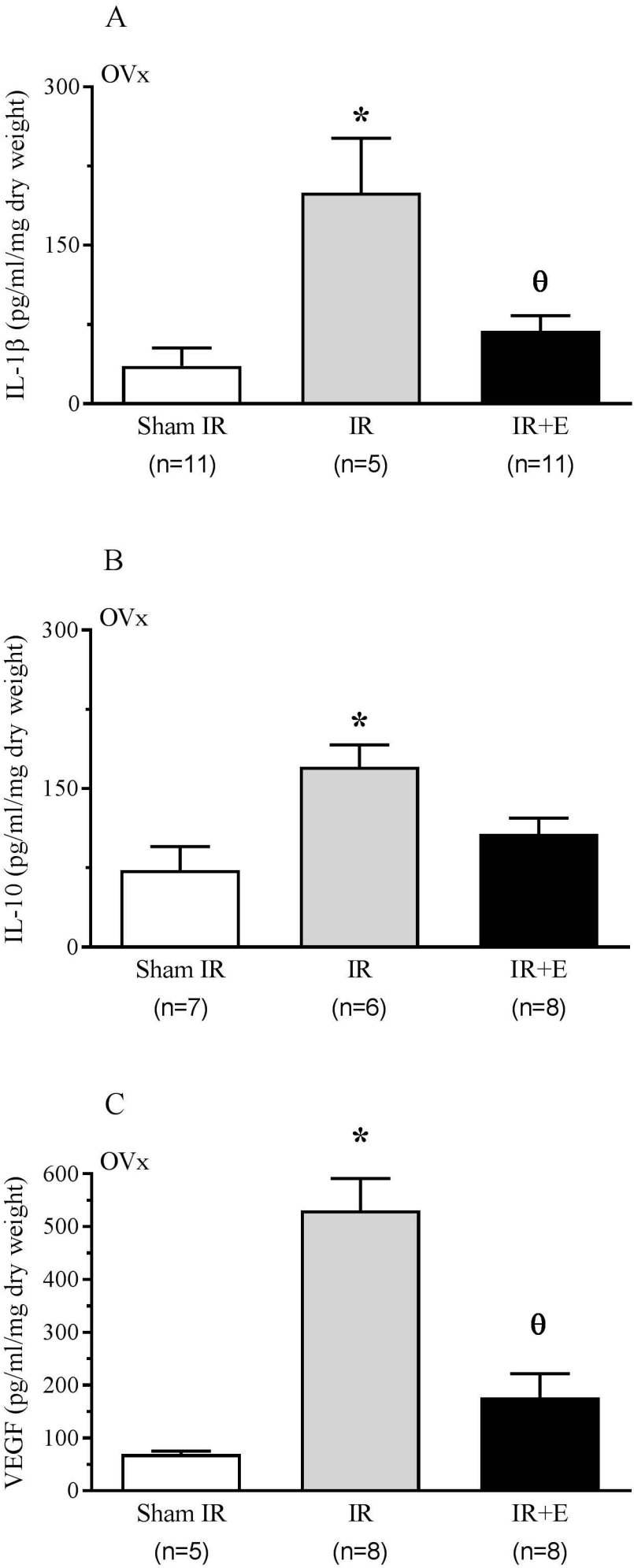

Figure 2 Fantozzi et al., 2017